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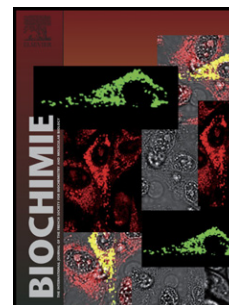
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Highlights

- Novel chymotrypsin-like activity implicated in the maintenance of cell viability
- Inhibition by TMR-Phe^P(OPh)₂ caused a decrease in HeLa and U251 cell viability
- Reduced viability was associated with an increase in caspase-3 activity over time
- PARP cleavage and phosphatidylserine translocation were also observed
- Inhibition of this protease initiates a cascade of apoptotic-related events

Chymotrypsin-like Serine Proteinases are Involved in the Maintenance of Cell Viability

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Abstract

An increasing number of studies have implicated serine proteinases in the development of apoptosis. In this study, we assessed the ability of a set of highly specific irreversible inhibitors (activity probes), incorporating an α -amino alkane diphenyl phosphonate moiety, to modulate cell death. In an initial assessment of the cellular toxicity of these activity probes, we discovered that one example, *N*- α -tetramethylrhodamine phenylalanine diphenylphosphonate {TMR-Phe^P(OPh)₂} caused a concentration-dependent decrease in the viability of HeLa and U251 mg cells. This reduced cell viability was associated with a time-dependent increase in caspase-3 activity, PARP cleavage and phosphatidylserine translocation, establishing apoptosis as the mechanism of cell death. SDS-PAGE analysis of cell lysates prepared from the HeLa cells treated with TMR-Phe^P(OPh)₂, revealed the presence of a fluorescent band of molecular weight 58kDa. Given that we have previously reported on the use of this type of activity probe to reveal active proteolytic species, we believe that we have identified a chymotrypsin-like serine proteinase activity integral to the maintenance of cell viability.

Keywords: Chymotrypsin, proteinases, apoptosis, serine, inhibitor, diphenylphosphonate

Abbreviations: PARP – Poly (ADP-ribose) polymerase, PS – phosphatidylserine, TMR-Phe^P(OPh)₂ – *N*- α -tetramethylrhodamine phenylalanine diphenylphosphonate; TLCK - *N*- α -tosyl-L-lysine chloromethyl ketone , TPCK - *N*- α -tosyl-L-phenylalanine chloromethyl ketone,

1. Introduction

Proteolytic cleavage of intracellular proteins is a core feature of apoptosis. In this regard, the role of the caspase family of cysteine proteinases has been well-established; however, increasing attention is now being paid to the potential roles of other protease classes and families in this process. The involvement of serine proteinases in apoptosis was suggested as early as 1987 [1] and by 1994, a further association was established when it was demonstrated that the introduction of chymotrypsin and trypsin into tumour cells led to a form of cell death indicative of apoptosis [2]. Other studies have focussed on attempts to identify and describe the actual role and function of serine proteases within apoptotic processing [3-7] however; the specific proteinases involved remain elusive and unidentified. Chymotrypsin-like proteases have however, emerged as prime candidates for further study [8-11].

Due to the diversity and quantity of proteases involved in apoptosis, experimental tools in the identification of unknown proteases within model systems have centred on the utilisation of protease inhibitors which facilitates the indirect study of apoptotic effects through the modulation or augmentation of particular apoptotic events. Protease-inhibitor binding alone however, does not impart information about the location, quantity or subcellular identification of target proteases. In addition, classification of an enzyme as belonging to the serine proteinase class on the basis of inhibition by *N*-Tosyl-Phenylalanine chloromethyl ketone (TPCK) and *N*-Tosyl-Lysine chloromethyl ketone (TLCK) should be treated with circumspection, as these compounds are not uniquely diagnostic for the action of this mechanistic class [12-14].

In contrast, Powers and coworkers have pioneered the development of potent and peptide derivatives of diphenyl [1-(*N*-peptidylamino)alkyl] phosphonate esters that act as exquisitely selective, irreversible inactivators of the serine proteases [15-17]. This group utilized biotinylated and fluorescently labelled derivatives to detect a range of serine proteolytic activities, including lymphocyte serine proteases (granzymes) [18-19]. We have also reported on the application of such phosphonate-based inhibitors for the detection of trypsin-like and chymotrypsin-like serine proteases [20-22] and, more recently, on the development of an active site-directed

probe for dipeptidyl peptidase IV-like serine proteases, based on a biotinylated dipeptide proline diphenylphosphonate [23-24].

Active site-directed inhibitors consisting of a warhead, a specific amino acid sequence and a reporter group (e.g. biotin) are essential tools in the application of the detection and characterisation of proteolytic activities in various biological systems [20, 25, 26]. In addition to biotinylated affinity labelling, the use of fluorogenic labels has been developed as another primary method of proteinase visualisation [22, 27]. This latter approach in particular, accommodates confocal and fluorescent microscopy as well as flow and laser cytometry, resulting in enhanced imaging and measurements of intracellular activity [28].

Based on the emerging research implicating serine proteases within apoptotic pathways, we investigated the presence of novel serine proteases utilising a combination library of 'in-house' and commercially available inhibitors. This approach has recently been adopted by other research groups [29]. The majority of 'in-house' inhibitors utilised in our study were based on the diphenyl phosphonate war-head. Diphenyl phosphonate groups are specific and potent irreversible inhibitors of serine proteases [15, 30] reacting covalently with the active site serine, thus affording a phosphorylated and irreversibly inhibited enzyme [16-17]. Diphenyl phosphonates are advantageous over other synthetic serine protease inhibitors as they show no activity against cysteine proteases, are non-toxic *in vivo* and are exceptionally stable under physiological conditions [15, 31-32]. Utilising the specific affinity probe TMR-Phe^P(OPh)₂, we report the detection of a novel chymotrypsin-like serine proteinase, inhibition of which initiates caspase-mediated cell death *in vitro*. We therefore, postulate a critical role for this enzyme in the maintenance of cell viability, although the identification of the protein substrate has yet to be determined.

2. Material and Methods

2.1 Cell culture and reagents: HeLa cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, UK) and cultured in Eagles Minimum Essential Medium (EMEM) (Sigma-Aldrich Co. Ltd, Irvine, UK) supplemented with L-Glutamine (2 mM), Penicillin (10,000 units/ml) / Streptomycin (10,000 µg/ml streptomycin sulphate in 0.85% saline) and 10%

(v/v) Foetal Calf Serum (FCS) (Biowest, UK). The U251 mg cell line (American Type Culture Collection, Rockville, MD) was derived from a human glioblastoma multiforme. The cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 mmol/L glutamine and 10% (v/v) foetal calf serum (FCS). With the exception of EMEM and FCS, all cell culture media and reagents were purchased from Gibco BRL, Paisley, Scotland.

2.2 Inhibitor/affinity probe synthesis: The labelled diphenylphosphonate derivatives of phenylalanine were synthesised within the Biomolecular Sciences group, School of Pharmacy, Queen's University, as previously described [22] utilising a modification of the method of Oleksyszen et al. [15]. N- α -tosyl-L-phenylalanine chloromethylketone (TPCK) and the cell-permeable caspase-3 inhibitor, Biotin-DEVD-FMK were purchased from Sigma Chemical Company, Poole, England.

2.3 Cell viability studies: Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (Sigma Chemical Company, Poole, England). In brief, cells were seeded onto a sterile 96-well cell culture plate at a concentration of 1×10^5 and incubated overnight at 37°C. After 24 hours media was aspirated from cells and 100 μ l fresh media containing the peptide treatments added. Cells were further incubated at 37°C for the appropriate time period, followed by the addition of MTT (10 μ l of a 10 mg/ml aqueous solution). Solubilisation of the resultant formazan crystals was through the addition of dimethyl sulphoxide (DMSO) and colour intensity was measured quantitatively at 550 nm on a Tecan Spectra Thermo plate reader from Tecan (Austria).

2.4 Enzymatic activity assays: Following incubation with 100 μ M Tetramethylrhodamine labelled-Phe^P(OPh)₂ (henceforth known as TMR-Phe-DPP), cells were harvested and disrupted using lysis buffer (0.1% (v/v) Triton X-100 in sterile PBS). Caspase-3 and chymotrypsin-like activity were measured in lysates (25 μ g protein/100 μ l assay) utilising a standard microplate assay under the following conditions: Caspase-3; 50 mM HEPES, 100 mM NaCl, 0.1% (w/v) CHAPS, 10 mM DTT, 10% (w/v) sucrose containing Ac-DEVD-AMC (Calbiochem, CA, USA) (final concentration 25 μ M) and for chymotrypsin-like activity; 50 mM sodium phosphate buffer (pH 7.4) containing N-Succinyl-Ala-

Ala-Pro-Phe-AMC (Sigma-Aldrich, St. Louis, USA) (final concentration 50 μ M). The rate of substrate hydrolysis was monitored every 60 s over a period of 1 hr using a FLUOstar OPTIMA (BMG Labtech) (λ_{ex} 380 nm and λ_{em} 460 nm).

2.5 PARP cleavage Assays: Following treatment, cells were harvested and lysed as described in 2.4. Lysates were reduced and denatured prior to electrophoresis by incubation with Laemmli treatment buffer at 100°C for 10 minutes. Equal amounts of protein (25 μ g), were subjected to SDS-PAGE before being electroblotted onto a nitrocellulose membrane. Non-specific binding sites were blocked by overnight incubation at 4°C with PBS containing 0.1% (v/v) Tween-20 (PBST) and 3% (w/v) BSA. Immunodetection was performed using a PARP monoclonal antibody (1:500 dilution; BD Pharmingen, San Diego, USA) in PBST containing 1% (w/v) BSA followed by washing in PBST containing 1% BSA (w/v) and incubation with a secondary peroxidase-coupled goat anti-mouse antibody (1:10000 dilution; BioRad, Herts, UK) under the same conditions. Blots were developed using the Supersignal West Pico Chemiluminescence system (Pierce, Rockford, IL, USA).

2.6 Phosphatidylserine translocation and nucleic acid staining: HeLa cells (1×10^5) were seeded onto a sterile 96-well cell culture plate and incubated overnight at 37°C to allow for detachment. At appropriate timepoints, media was aspirated from cells and 100 μ l fresh media containing the peptide treatments added. Staining of phosphatidylserine translocation was undertaken using Annexin V-Cy5 Apoptosis Detection Kit according to the manufacturer's instructions (Biovision, CA, USA). Simultaneously, cells were stained with SYTOX® Green Nucleic Acid Stain according to the manufacturer's protocol (Molecular Probes, U.S.A.). Visualisation and imaging of cells was undertaken using an ECLIPSE TE300 inverted microscope with a TE-FM Epi-Fluorescence attachment and DXM1200 digital still camera (Nikon).

2.7 Confocal imaging of cells: U251 mg or HeLa cells were seeded at a concentration of $2 \times 10^5 \text{ ml}^{-1}$ onto a 22 mm x 22 mm coverslip within a 6 well sterile cell culture plate and incubated at 37°C overnight to aid attachment. Media was aspirated, and cells washed twice in sterile PBS. Media (3 ml) containing various concentrations of TMR-Phe-DPP (100 μ M, 10 μ M, 1 μ M and 100 nM) was added and cells reincubated for 1 hour. Media containing peptide treatments were subsequently removed and cells washed with sterile PBS to remove residual

staining of the fluorophore. Coverslips were mounted onto microscope slides using mounting media and images taken using a Leica TCS SP2 Confocal microscope.

2.8 Visualisation of protease target by SDS-PAGE: HeLa cells were incubated at 37°C, 5% CO₂ for 24 hours with 100 µM TMR-Phe-DPP and 100 µM BPS-Phe-DPP. Cells were lysed in PBS, containing 0.1% (v/v) Triton X-100) and subjected to SDS-PAGE as mentioned previously. As an alternative, HeLa cells were incubated for 30 min with 100 µM Bio-Phe-DPP, TMR-Phe-DPP and Z-Phe-DPP. Cells were then lysed as before and the lysate subjected to a secondary incubation with 100 µM Biotin-Phe-DPP. The samples were then subjected to SDS-PAGE and Western Blotting with visualisation using streptavidin-horseradish peroxidase (1:5000) and chemiluminescence as before.

2.9 Statistical analysis: Data was analysed were appropriate using the Mann-Whitney U test of significance.

3. Results and Discussion

3.1 Cell viability studies

In our initial studies we assessed the effect of an extensive library of synthetic serine proteinase inhibitors on the viability of HeLa cells. These included inhibitors directed against elastase-like, trypsin-like and chymotrypsin-like proteases. This screen was carried out using 100 μ M of each inhibitor with an exposure time of 24 hours. Of the inhibitors tested, only the chymotrypsin-directed inhibitors exhibited consistent and significant effects on viability (Table 1). Two of the phenylalanine diphenyl phosphonate derivatives possessed the fluorescent labels EDANS and TMR, whilst another was biotinylated (BPS-Phe-DPP). Inclusion of a tosyl-labelled phenylalanyl diphenyl phosphonate was to enable direct comparisons to be made with the traditionally used, commercially available TPCK. With the exception of the biotinylated inhibitor (BPS-Phe-DPP), each of the diphenyl phosphonates tested caused a significant reduction in cell viability of at least 30%, with the tetramethylrhodamine derivative exhibiting the most pronounced loss. It is postulated that the biotinylated inhibitor, which also possesses a PEG-succinyl-linker group, could have been too hydrophilic to cross the plasma-membrane. The commercially available chloromethylketone inhibitors, TPCK and TLCK have traditionally been utilised as ‘diagnostic tools’ to establish the action of serine proteinases. In this study, we compared the effect of TPCK with its exactly analogous diphenyl phosphonate, Tosyl-Phe-DPP. We found that the former caused a 20% loss in cell viability compared to a near 30% decrease brought about by the latter.

TPCK, originally designed as an irreversible inhibitor of chymotrypsin has however, been found to impact on apoptosis in a variety of ways which may not be due to its inhibition of serine proteases e.g. TPCK causes the release of pro-apoptotic factors from the mitochondria, can act as efficient direct inhibitors of mature caspases and may interfere with the cell cycle [33, 34]. Activation of apoptosis by TPCK also appears to be dependent upon whether apoptotic stimulation is present [33-35]. We found that when co-incubated with staurosporine, an apoptotic inducer, TPCK had a protective effect on HeLa cell viability (data not shown), which is in agreement O’Connell et al [36] and King et al [33]. However, in the absence of staurosporine TPCK induced a loss of viability. The wide range of biochemical and cellular effects displayed by chloromethylketones, including their direct inhibition of mature caspases

[34] indicates that the diphenylphosphonate-based inhibitors may be more selective tools to elucidate the role of serine proteases in apoptosis.

TMR-Phe-DPP was found to exert a dose-dependent reduction in the viability of HeLa cells when studied over a range of 24 hr periods up to 96 hrs, with significant effects apparent at concentrations of 25 μ M and above. To discount possible cytotoxicity due to compound-related differences (such as the inclusion of the TMR fluorophore) as a possible cause of cell death, we also carried out a dose response curve in HeLa cells with the TMR fluorophore alone for time periods of 24-96 hours. No discernible cytotoxic effects were observed in these studies (data not shown). The relative sensitivity of HeLa to TMR-Phe-DPP (100 μ M) over time indicated a loss of viability within 4 hours of treatment (5%), increasing to 61% at 12 hours followed by a rapid loss of viability between 12 and 24 hours with only 9% of cells remaining. In comparison, U251 mg cells demonstrated a high level of resilience to loss of viability in the early time-points, with 94% of cells still viable at 12 hours. By 18 hours 64% of cells remained viable. A rapid decline then ensued and by 24 hours viability was 12%, a figure comparable with that observed with HeLa cells. This data suggests that incubation with 100 μ M TMR-Phe-DPP has a negative impact on the viability of both HeLa and U251 mg cells. To clarify that TMR-Phe-DPP was targeting a chymotrypsin-like protease, HeLa cells were incubated with 100 μ M of the inhibitor for various time-points. Cells were harvested and lysed before being assayed fluorogenically for chymotrypsin-like activity (Figure 1). No significant differences were found between control and treated cells at early time-points however, a significant reduction in intracellular activity was observed at 4 and 6 hours with p-values of 0.0183 and 0.0487 respectively. At 12 hours the differences were highly significant (***; $p < 0.001$). Intra-plate variation may have impacted on a loss of significance at some of the other time-points.

3.2 Analysis of caspase-3 activity

Detection and measurement of activated caspase-3 is one of the classic benchmarks for confirming that apoptosis has been activated in cells [37]. Caspase activation initiates the irreversible state of apoptosis, hence detection of this event is often critical and a variety of approaches have been developed towards this aim [28]. Detection methods can be direct, for example immunodetection utilising a specific caspase-3 antibody or indirect through substrate-based activity assays.

Caspase-3 activity assays were performed on cell lysates prepared from HeLa and U251 mg cells exposed to 100 μ M TMR-Phe-DPP for periods of up to 12 hours. Activity was monitored using the fluorogenic substrate Ac-DEVD-AMC. An increase in active caspase-3 levels over time, caused by the exposure of HeLa and U251 mg cells to TMR-Phe-DPP, is shown in Figures 2A and B, respectively. TMR-Phe-DPP was observed to induce a significant activation of caspase-3 after 4-6 hours of exposure. By 24 hours, levels of the protease increased 3.5-fold in the HeLa cells ($p < 0.05$) and 10-fold in the U251 mg cells ($p < 0.001$) compared to the no treatment control. The levels and timeliness of caspase-3 activation correlated appropriately with the loss of viability observed in both cell lines upon treatment with TMR-Phe-DPP.

To further ascertain that the loss of viability induced by TMR-Phe-DPP was caspase-3 dependent and therefore associated with apoptosis, cells were pre-incubated for 4 hours with 10 μ M of the cell-permeable caspase-3 inhibitor Biotin-DEVD-FMK, followed by incubation with the TMR-labelled peptide as before. Cell viability was measured using the MTT assay. Pre-incubation with the caspase-3 inhibitor served only to stall the initiation of apoptosis. From 18 hours, cell viability was reduced by over 90% both in the presence and absence of Biotin-DEVD-FMK.

3.3 PARP cleavage Assays

Caspase-3 cleavage of the nuclear repair enzyme poly (ADP-ribose) polymerase (PARP) is considered a very sensitive marker of caspase activation and apoptosis [38-39]. In non-apoptotic cells PARP is observed in the uncleaved form (116 kDa), whereas in apoptotic cells PARP is characteristically cleaved to an indicator apoptotic fragment (85 kDa). To provide further confirmation that the loss of cell viability was due to apoptosis, cells were incubated with 100 μ M TMR-Phe-DPP for a period of 30 minutes to 24 hrs. In both HeLa and U251 mg cell lines, a residual amount of PARP cleavage was observed, however this is proposed to be due to normal housekeeping duties and no observable differences between control and treated cells were detected between 30 minutes and 8 hours. For HeLa cells following 12 hour post-treatment with TMR-Phe-DPP there was, however, a total depletion of the 116 kDa species, and only the 85 kDa form was visible (Figure 3A). In contrast, lysates prepared from U251 mg cells, which showed a delayed reduction in viability when treated with the diphenyl phosphonate inhibitor, revealed significant levels of the higher molecular

weight form of PARP in addition to the processed 85 kDa species at 12, 18 and 24 hours post treatment times (Figure 3B).

3.4 Phosphatidylserine Translocation

Annexin V is a small Ca^{2+} -dependent protein with high affinity for phosphatidylserine (PS) [40]. In normal living cells, PS is located in the inner layer of the cell membrane only, but in apoptotic cells this phospholipid is translocated to the outer leaflet. Once on the cell surface, PS can be easily detected by staining with a fluorescent conjugate of Annexin V and detection analysed by flow cytometry or fluorescent microscopy. To observe whether TMR-Phe-DPP would induce this translocation, HeLa cells were grown on coverslips and incubated with 100 μM of the inhibitor for various time-periods. Using the Annexin-V-Cy5 staining kit, visualisation of PS was obtained using confocal microscopy (Figure 4). Translocation of PS was observable at 3 hours with diffuse staining clearly visible at 4 and 6 hours. In conjunction with annexin-V staining, Sytox Green, a nucleic acid stain, was used to observe necrotic and late apoptotic cells, based on the principle that nucleic acid stains easily penetrate cells with compromised plasma membranes. There was no marked increase in Sytox green stained cells between the treated group and the control, indicating that the cell death observed was due to apoptosis and not necrosis (data not shown).

3.5 Transport and uptake of TMR-Phe-DPP into cells

Previous results based on cell viability and caspase-3 activation suggested that TMR-Phe-DPP is cell-permeable. Studies were undertaken to observe the time-course of this inhibitor in relation to transmembrane delivery and subsequent morphological cellular changes. In addition to observing its intracellular location, it was important to understand the mechanism by which it enters cells.

The microscopy images of HeLa cells showed that the inhibitor started to concentrate intracellularly within 30 minutes (Figure 5). By 4 hours post incubation morphological changes relating to apoptosis were observed, in particular the rounding up of cells. By 8 hours, blebbing of the plasma membrane alongside increased 'rounding up' of cells was apparent indicative of apoptotic morphological changes.

Confocal imaging of U251mg cells after treatment with TMR-Phe-DPP (100 μM) clearly showed a concentration of fluorescence within the cytosol with potential vesicle involvement (Figure 6A and B). This was further apparent at a peptide

concentration of 1 μ M which highlighted the efficiency of transport and localisation of this inhibitor (Figure 6C).

3.6 Identification of Molecular Weight of the Protease Target of TMR-Phe-DPP

It is clear that TMR-Phe-DPP when incubated with cells induces loss of viability through the initiation of a cascade of apoptotic events. The target protease may therefore have a critical role to play in the maintenance of cell viability and could have great potential as a target for anti-cancer therapies. To ascertain the molecular weight of its target protease, HeLa cells which had been treated with TMR-Phe-DPP were lysed and subjected to SDS-PAGE. A single fluorescent band of 58 kDa was detected under UV (Figure 7A). This species was also detected in cell lysates using BPS-Phe-DPP with the band markedly reduced/absent in cell preparations which had been pre-incubated with TMR-Phe-DPP and Z-Phe-DPP, respectively prior to lysis (Figure 7B). This confirmed the target of these inhibitors, regardless of protecting/reporter group, to be a chymotrypsin-like protease and highlights the relative cell permeability of these compounds.

4. Conclusion

A core feature of apoptosis is the proteolytic cleavage of intracellular proteins [41]. The proteases involved and the role they play will be variable and dependent upon both the origin of apoptotic stimuli and the cell type involved [42]. Once the process of apoptosis is initiated cells undergo a series of morphological and biochemical changes including membrane blebbing, caspase-3 activation, phosphatidylserine translocation and PARP cleavage [43]. Our initial investigations established that a chymotrypsin-like activity had a protective role in cell viability, as inhibition led to cell death within 24 hours. One inhibitor in particular, the fluorescently labelled TMR-Phe-DPP, had a profound effect on cell death. In light of much published research into the role of serine proteases in apoptosis, proof that the observed cell death was apoptosis and not necrosis was paramount. Further biochemical investigations using this very effective inhibitor confirmed an apoptotic mechanism of cell death as in addition to a time-dependent increase in caspase-3 activity; cleavage of the DNA repair enzyme PARP and translocation of phosphatidylserine to the outer leaflet of the plasma membrane were observed. Exclusion of sytox green, a nucleic

acid stain also confirmed that the observed cell death was apoptotic and not due to necrosis.

These results highlight the importance of chymotrypsin-like serine proteases in the maintenance of cell viability.

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Figure Legends

Figure 1. HeLa cells were incubated with 100 μ M TMR-Phe-DPP, for various timepoints before being harvested and lysed in sterile PBS containing 0.1% TX-100. Lysate containing 25 μ g protein was assayed for chymotrypsin activity utilising the fluorogenic substrate *N*-Succinyl-Ala-Ala-Pro-Phe-AMC, final concentration 50 μ M. Significant differences are denoted by *.

Figure 2. HeLa (A) and U251 mg (B) cells were incubated with 100 μ M TMR-Phe-DPP for various timepoints before being harvested and lysed in sterile PBS containing 0.1% (v/v) TX-100. Lysates containing 25 μ g protein were assayed for caspase-3 activation utilising the fluorogenic substrate Ac-DEVD-AMC, final concentration 25 μ M. No significant difference was found between control and treated cells within each timepoint between 30 min and 3 hours. A significant difference (*) was observed 6 and 4 hrs, respectively with a highly significant (***) difference observed at 12 hrs for U251 mg cells ($p < 0.0001$).

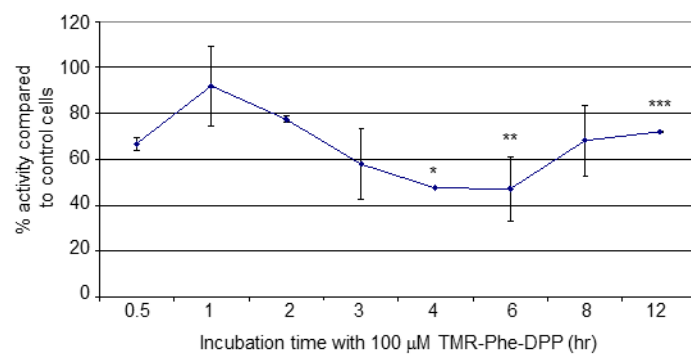
Figure 3. HeLa (A) and U251 mg (B) cells were incubated with 100 μ M TMR-Phe-DPP, for various timepoints between 30 minutes and 24 hours. Cells were then harvested and lysed with PBS containing 0.1% (v/v) TX-100. PARP cleavage was detected in samples (25 μ g protein) by immunodetection utilising an anti-PARP antibody (1:500) and a goat anti-mouse HRP conjugate (1:10,000). Arrows represent the uncleaved (116 kDa) and cleaved (85 kDa) PARP species.

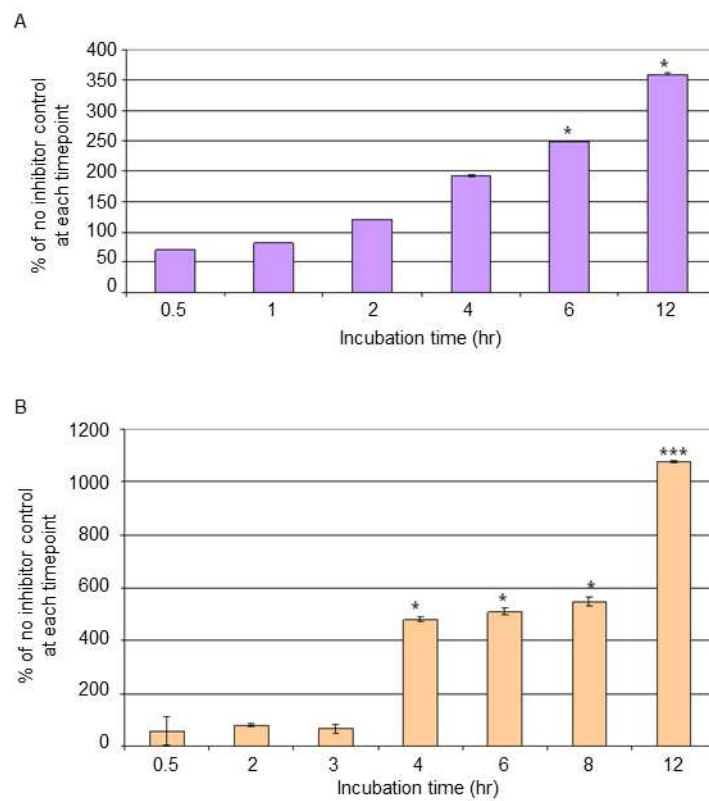
Figure 4. HeLa cells were incubated with 100 μ M TMR-Phe-DPP, for various timepoints: (A) 1 hr; (B) 3 hr; (C) 4 hr and (D) 6 hr, before staining with Annexin V-Cy5 Apoptosis Detection Kit according to the manufacturer's instructions. All images are at x200 magnification.

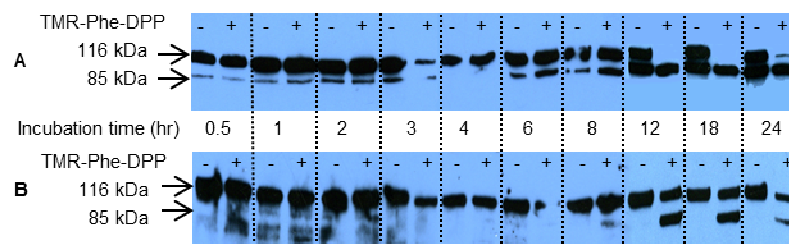
Figure 5. Phase contrast and fluorescent overlay images of HeLa cells following incubation with 100 μ M TMR-Phe-DPP: (A) 30 min; (B) 1 hr; (C) 4 hr and (D) 8 hr. The inhibitor was observed to localise in the cytosol within 30 minutes. At 4 hours post incubation, morphological changes indicative of apoptosis were apparent (Magnification x200).

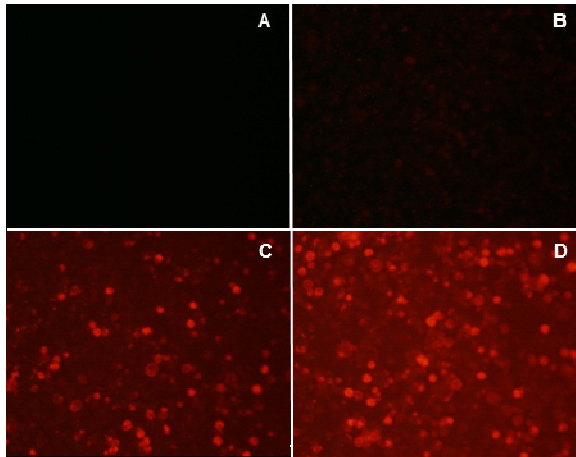
Figure 6. Confocal microscopy images of U251 mg cells following 1 hour incubation with 100 μ M TMR-Phe-DPP. Cells were grown on coverslips before the addition of the inhibitor. Following 1 hour, cells were washed and mounted onto microscope slides. Images show cytosolic location of the peptide (A) x2500 and (B) x4000 magnification. (C) 1 μ M peptide; x7000 magnification.

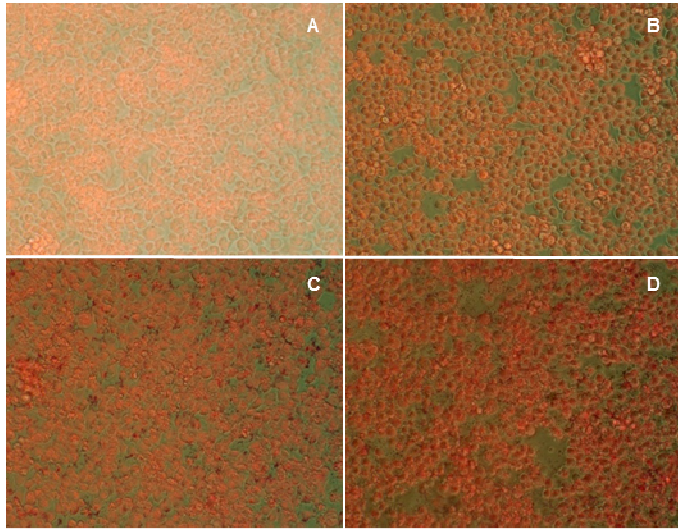
Figure 7. A. Intact HeLa cells were incubated at 37°C, with 5% CO₂ for 24 hours with 100 μ M TMR-Phe-DPP and 100 μ M BPS-Phe-DPP. Cells were lysed before undergoing SDS-PAGE. UV light highlighted a fluorescent band of approximately 58 kDa in lane 1, which contained the lysate and TMR-Phe-DPP. Lanes: (M) markers; (C) untreated control cells; (1) HeLa cells + TMR-Phe-DPP and (2) HeLa cells + BPS-Phe-DPP. The fluorescence at the bottom of lane three is attributed to ‘wash-through’ of the fluorogenic probe. B. Intact HeLa cells were incubated at 37°C for 30 min with (1) control (no probe); (2) Bio-Phe-DPP (not thought to be cell permeable); (3) TMR-Phe-DPP (cell permeable) and (4) Z-Phe-DPP (cell permeable). Cells were then lysed and the lysate subjected to a secondary incubation with 100 μ M Biotin-Phe-DPP. The samples were then subjected to SDS-PAGE and Western Blotting with visualisation using streptavidin-HRP (1:5000).

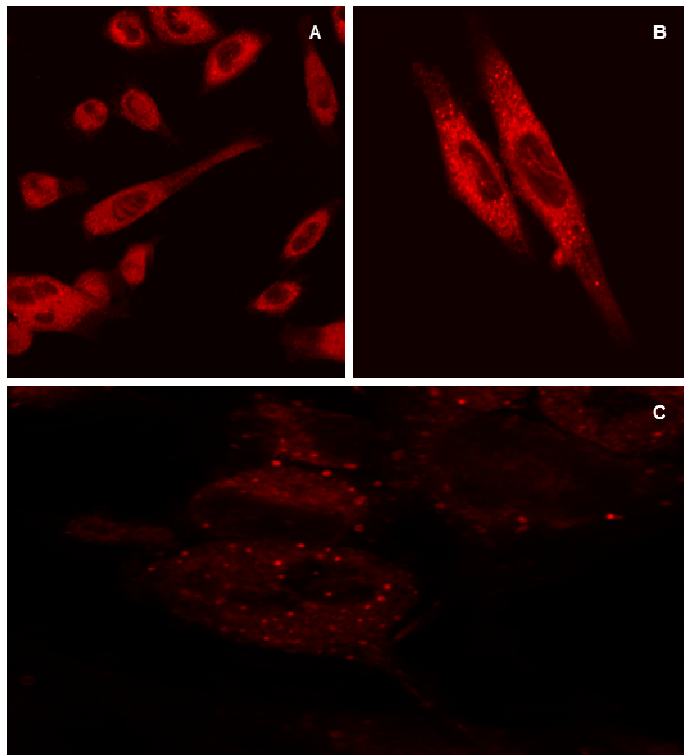












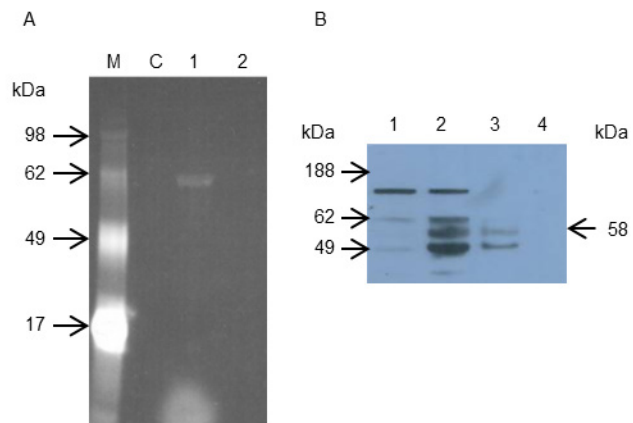


Table 1.

HeLa cells were incubated for 24 hours at 37°C with 100 µM of chymotrypsin-like proteinase inhibitors. Cell viability was assessed using standard MTT assay and presented as % cell viability of control

Inhibitor	Cell viability (% control)	p-value
Z-Phe-DPP	67%	0.0002***
EDANS-Phe-DPP	73%	0.0006***
BPS-Phe-DPP	94%	0.1957
TMR-Phe-DPP	16%	<0.0001***
TPCK	79%	0.0181*
Tosyl-Phe-DPP	71%	<0.0001***

Z-Phe-DPP: Benzyloxycarbonyl-phenylalanyl-diphenylphosphate; EDANS: 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid; BPS: Biotin-PEG-Succinyl; TMR: Tetramethylrhodamine and TPCK: N- α -tosyl-L-phenylalanine chloromethylketone.